A platform approach to purification of antibody fragments

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Antibody fragments constitute a promising class of biopharmaceutical products. As compared with full length antibodies, their fragments have unique properties that make them favorable for certain therapeutic conditions. However, due to their high molecular diversity, the use of a purification platform approach (i.e., standard sets of unit operations, conditions, and methods applied to a given class of molecules) is more difficult for fragments than for full length antibodies, for which the Fc region can be utilized as a common binding motif. However, with recent developments of novel affinity chromatography resins, there are new emerging possibilities. Here, we present affinity chromatography resins that offer the possibility of a platform approach to the purification of the majority of antibody fragments. The described BioProcess™ resins offer high selectivity and excellent pressure-flow properties for high purity and yield in industrial-scale purifications. In addition, several case studies on purification of antibody fragments are described.

Introduction

Over the last 20 years, monoclonal antibodies (mAbs) have shown success in many therapeutic areas. While offering efficient treatment of several major diseases, such as breast cancer, some functional limitations of antibodies have been observed. Their large size, for example, can prevent tumor-specific antibodies from efficient penetration and retention in the target tissue (1).

However, the multi-domain structure of antibodies supports the creation of smaller fragments that include the antigen binding domain (Fig 1). Antibody fragments (e.g., Fab, scFv, dAb) possess some advantageous properties suitable for a range of diagnostic and therapeutic applications. For example, fragments are smaller than mAbs (Fig 1), and thus can more easily penetrate tissues. As most antibody fragments are non-glycosylated, they can be produced in microbial cells, rather than the more costly mammalian cell culture processes upon which mAb manufacturers are dependent. Hence, following on the success of mAbs, antibody fragments are gaining increased interest as a protein-based biotherapeutics, and several have been approved for therapeutic use (2).

The methodologies, with which antibody fragments have been generated, range from proteolytic cleavage to modern genetic engineering approaches. Further diversification comes from antibody fragments being covalently or recombinantly enhanced with other functional properties. Antibody fragments have been linked with enzymes (3), toxins (4), and radionuclides for cancer treatment (5–7), so called antibody-drug conjugates (ADC). There are also examples where fragments have been linked with viruses for gene therapy, with liposomes or nanoparticles for improved drug delivery, and with dye or other sensing substances (8–12).

Types of antibody fragments

Fabs are considered the first generation of antibody fragments (13) and were initially generated by cleavage of an intact antibody using an enzyme, such as papain (14). Papain cleavage yields two monovalent Fab fragments, each composed of one variable heavy chain (V_H) and one variable light chain (V_L) linked by disulfide bonds and displaying a single antigen-binding site (Fig 2). Today, Fabs are produced using modern genetic engineering approaches.

The scFvs are monovalent structures, with affinity for a single antigen. With an approximate size of M_r 25 000, an scFv contains the variable regions of an antibody's heavy and light chains fused into a single polypeptide chain via a short flexible linker. An scFv comprises the complete antigen-binding site of its parental antibody molecule.

Consisting of the V_H or V_L domains, dAbs are some of the smallest functional antibody fragments that retain full antigen-binding specificity. The dAb is approximately one-tenth of the molecular weight of a normal antibody. Although dAbs contain only three of the six complementary determining regions from the parent antibody (Fig 3), they do exhibit antigen binding specificity and affinity. A dAb can be remarkably stable under harsh conditions of temperature, pressure, and denaturing chemicals (15).

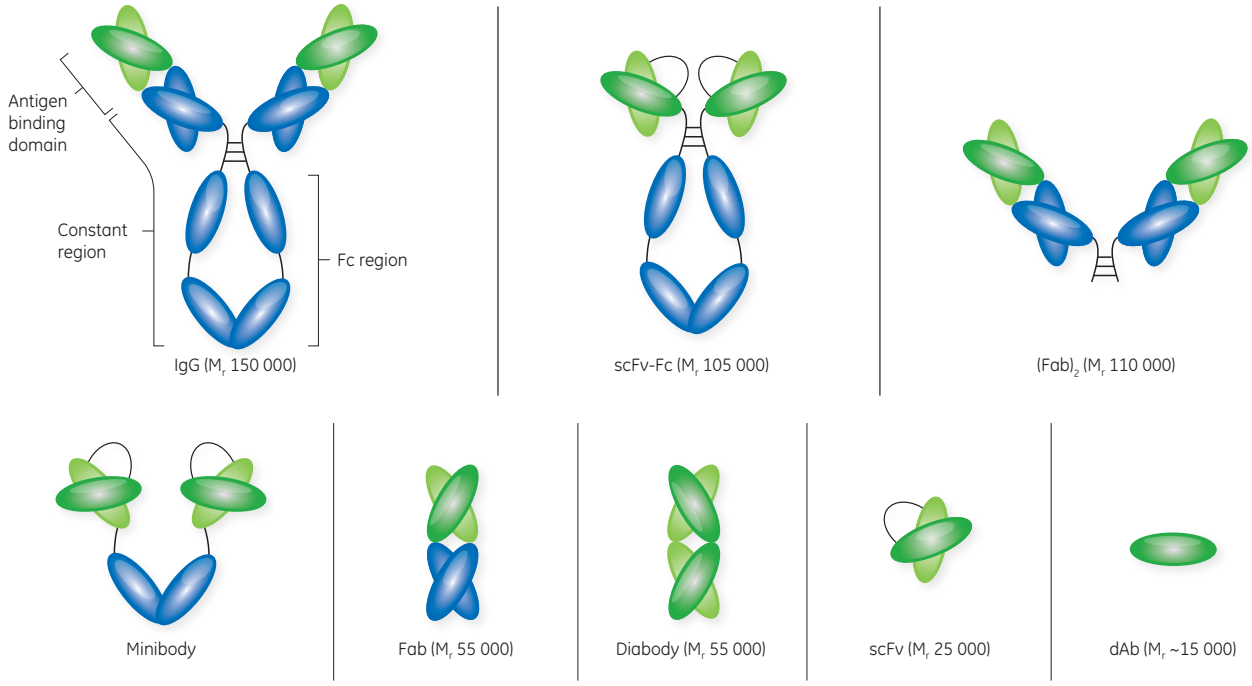


Fig 1. Structure of antibody and antibody fragments. Fab = fragment, antigen-binding; scFv = single-chain fragment, variable; and dAb = domain antibody.

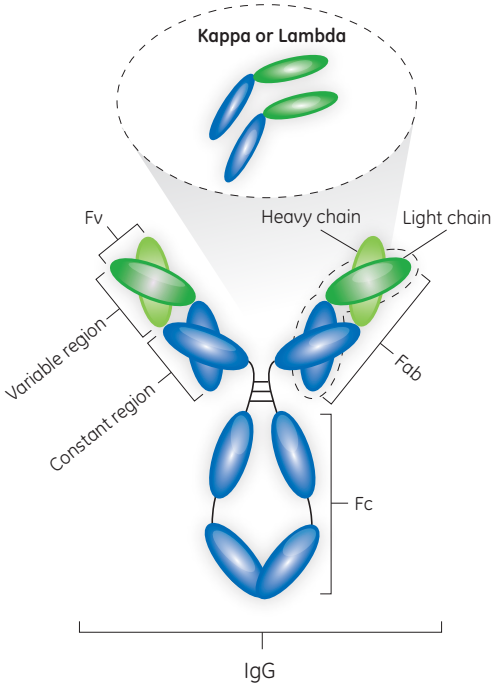


Fig 2. Antibody with marked domain names.

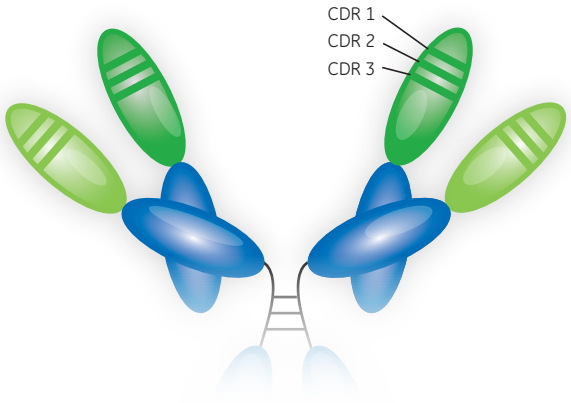


Fig 3. Complementarity determining regions (CDRs) are located in the variable domains and are the most variable parts of the antibody.

Purification platform approach

As a class of molecules, mAbs exhibit many shared properties that make them well-suited for a platform approach to downstream purification. The presence of an Fc region allows for a close-to generic purification approach using affinity chromatography methodology. Technology platforms allow for efficient processing from research and development, through clinical phase trials, to the manufacturing of the final product. Downstream mAb purification platforms commonly include a Protein A-based capture step followed by one or two polishing steps to remove remaining impurities. Protein A affinity resins offer high robustness and selectivity, often with more than 99% purity in a single step, which minimizes process development work.

Antibody fragments, on the contrary, are a more diverse group of molecules that lack the Fc region, making the purification of these targets more challenging. The development of specific, yet complex, purification protocols demands an extensive amount of time, money, and process development resources. Availability of platform alternatives for purification of antibody fragments would have further promoted their industrial production.

Production and purification of antibody fragments

Although higher eukaryotic cells have successfully been used to express antibody fragments, their small size and non-glycosylated nature allow the use of simpler and less costly prokaryotic or yeast expression systems. However, microbial expression systems place significant demands on culture clarification and the primary capture step.

Crossflow filtration (CFF) is a suitable technique for clarification of viscous or high-solid feeds such as microbial fermentation broth (16). Hollow fiber filter cartridges are commonly used for the CFF step. Because of their open channel structure, hollow fiber filters are well-suited for microfiltration applications such as recovery of proteins expressed in bacteria or yeast.

Several antibody fragments have been purified using cation exchange chromatography (CIEX) as the capture step. There are several commercial CIEX resins available that function over a broad range of conductivity and pH values. When an even wider range of operating conditions is required, resins providing more than one type of interaction between ligand and sample components (multimodal resins) can be used. For intermediate purification and final polishing, separation based on different selectivity than the primary technique (orthogonal) can involve anion exchange (AIEX) or hydrophobic interaction chromatography.

An ideal purification platform would be generically applicable to a wide range of antibody fragments, and allow rapid processing with high product purity and yield. While several processing options exist for antibody fragment purification, no methods of choice have yet emerged. The major drawback to non-affinity-based primary capture is that significant process development effort must be applied to each fragment under consideration.

Protein L affinity ligand for a broad range of antibody fragments

Protein L is present at the surface of about 10% of *Finegoldia magna* strains. Attributed to its rather unique binding specificities, Protein L offers options to purification of antibody fragments. Native Protein L is a M_r 76 000 to 106 000 protein, containing four or five highly homologous, consecutive extracellular domains responsible for the protein's interaction with Ig kappa light chains. Given that its target is the kappa light chain, Protein L will bind to representatives of most antibody classes, including IgG, IgM, IgA, IgE, and IgD (Table 1). It should be noted, however, that the native form of Protein L does not recognize antibodies (or related fragments) from certain animal species (18).

As the binding site for Protein L is located in the framework region 1 (a less variable region than the CDRs of the variable domain) of the kappa light chain, fragments derived from antibodies that have the kappa light chain can be purified using Protein L. As Protein L interacts with the kappa light chain, it has no immunoglobulin class restrictions. Hence, Protein L offers the potential of being a broadly useful, if not fully as general as Protein A, affinity ligand (19). Approximately 60% of mammalian IgG light chains are kappa chains, with the remaining 40% being lambda chains that lack binding sites for Protein L (17, 20).

Toolbox for industrial purification of antibody fragments

Protein L-based affinity chromatography resins for research applications have been commercially available for many years. With the introduction of the Capto™ L resin, however, the first opportunity for an industrial platform for the purification of antibody fragments emerged (21). With its recombinant Protein L ligand, Capto L is a BioProcess chromatography resin with a broad affinity for a range of antibody fragments of different sizes that contain kappa light chains. With its rigid base matrix, allowing for high flow rates and high productivity, as well as low ligand leakage, Capto L resin is well suited for large-scale manufacturing. The recommended cleaning-in-place (CIP) protocol for Capto L resin is the use of 15 mM NaOH for 15 min, allowing for approximately 100 cleaning cycles with > 90% remaining binding capacity. Due to the high-affinity binding of Protein L to the variable region of the kappa light chain, Capto L purifies conventional Fabs, scFv, and dAbs.

Figure 4 shows the dynamic binding capacity (DBC) at 10% breakthrough (Q_{B10}) of Capto L for four different antibody fragments. Note that, as the DBC is normally measured in mg/mL, the molecular weight of the target molecule is an important factor to consider. Table 2 presents the DBC in relation to the target's molecular weight and the corresponding molar binding capacity.

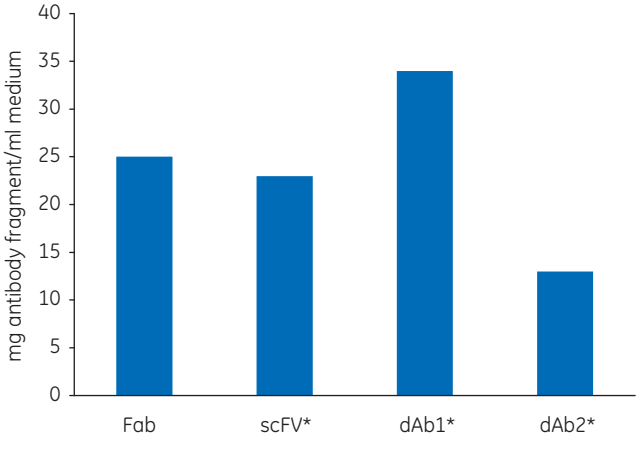


Fig 4. Q_{B10} of Capto L for four human antibody fragments. Fab fragment kindly provided by UCB Celltech. *Results obtained through customer collaborations.

Table 1. Protein L binding affinities (17)

Species	Antibody class	Affinity*
General	Kappa light chain (subtypes 1,3,4)	Strong
	Lambda light chain	No binding
	Heavy chain	No binding
	Fab	Strong
	scFv	Strong
	dAb	Strong
Human	IgG1	Strong
	IgG2	Strong
	IgG3	Strong
	IgG4	Strong
	IgA	Strong
	IgD	Strong
	IgE	Strong
	IgM	Strong
Mouse	IgG1	Strong
	IgG2a	Strong
	IgG2b	Strong
	IgG3	Strong
	IgM	Strong
Rat	IgG1	Strong
	IgG2a	Strong
	IgG2b	Strong
	IgG2c	Strong
Pig	Total IgG	Strong
Dog	Total IgG	Weak
Cow	IgG1	No Binding
	IgG2	No Binding
Goat	IgG1	No Binding
	IgG2	No Binding
Sheep	IgG1	No Binding
	IgG2	No Binding
Chicken	Total IgG	No Binding

* Binding to Protein L occurs only if the immunoglobulin has the appropriate kappa light chains. Stated binding affinity refers only to species and subtypes with appropriate kappa light chains. Lambda light chains and some kappa light chains will not bind.

Table 2. DBC of Capto L for four different human antibody fragments

Molecule	DBC (mg/mL)	M_r	Molar equivalence*
Fab	25	50 000	0.5 µmol Fab /mL resin
scFv fusion protein	23	57 000	0.5 µmol scFv/mL resin†
dAb1	34	25 000	1.4 µmol dAb/mL resin†
dAb2	13	10 000	1.3 µmol dAb/mL resin†

* As a comparison, note that typical Protein A resins capture approx. 0.3 µmol IgG/mL resin. Fab fragment kindly provided by UCB Celltech.

† Results obtained through customer collaborations.

LambdaFabSelect is an affinity resin used for the capture of Fabs containing the lambda light chain. LambdaFabSelect binds to the constant region of the lambda light chain and can therefore bind Fab fragments. Together, Capto L and LambdaFabSelect cover nearly all Fabs as well as a majority of the smaller antibody fragments. KappaSelect is an affinity resin that binds to the constant region on the kappa light chain and can be used to capture Fabs containing the kappa light chain under conditions where Capto L is found to be less suitable. The ligands of the LambdaFabSelect and KappaSelect resins are both based on single-chain antibody fragments with affinity for either human IgG lambda or kappa light chain. In addition to its binding in the Fc region, the recombinant Protein A ligand used in MabSelect™ affinity resin binds to the V_{H3} domain subtype of human IgG Fabs. Hence, MabSelect resin can be a useful alternative for capturing heavy chain dAbs that contain the V_{H3} domain subtype.

Figure 5 shows the affinity map of the four described affinity resins. All these resins are designed for industrial-scale manufacturing of antibody fragments. Based on a well-proven, high-flow agarose base matrix, these resins ensure high productivity and high dynamic binding capacity. In addition, all resins carry regulatory support and security of supply. Figure 6 displays a selection guide that illustrates when to use the different affinity resins of GE Healthcare's toolbox for the capture of antibody fragments.

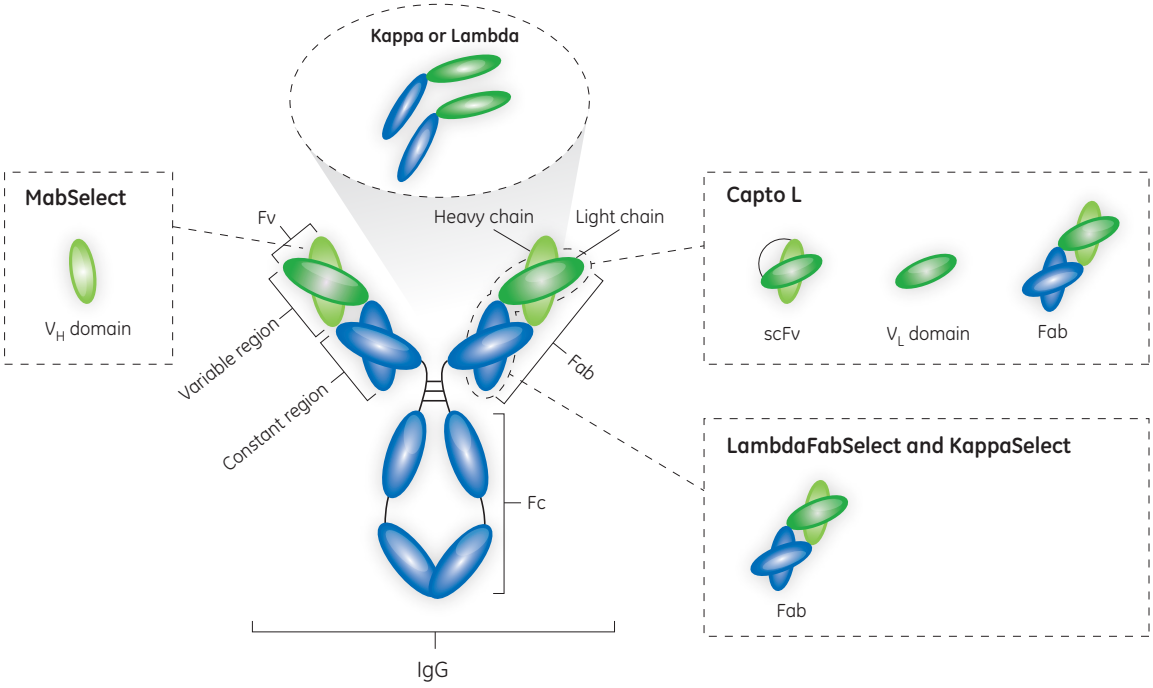


Fig 5. Affinity map for resins included in GE Healthcare's toolbox for the capture of antibody fragments.

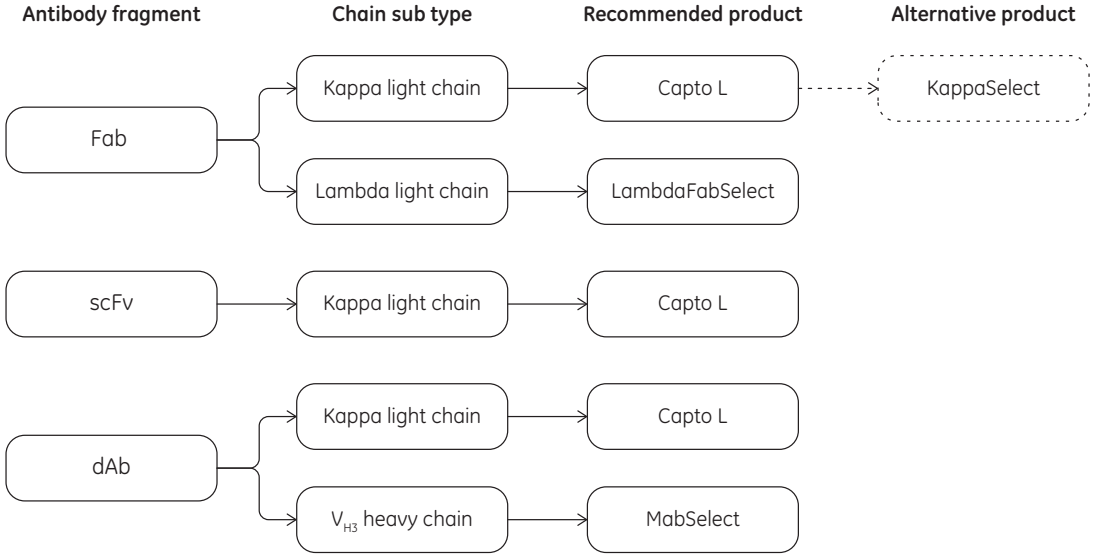


Fig 6. Selection guide of the capture toolkit

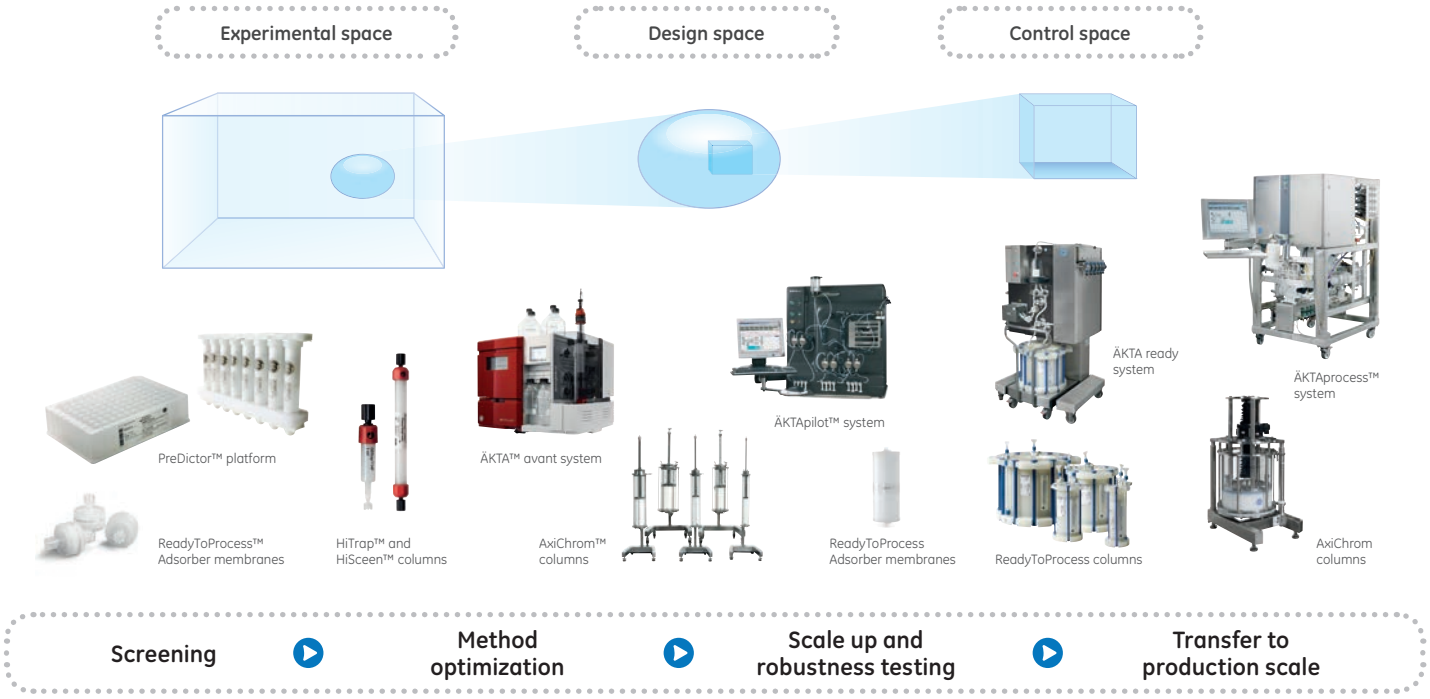


Fig 7. HTPD workflow including initial screening, verification, and further optimization of chromatography conditions in the purification of a target protein.

High-throughput process development (HTPD)

High throughput in process development is highly beneficial in research efforts for developing optimized and robust protocols for purification of antibody fragments (Fig 7). By the introduction of HTPD tools, significant gains in efficiency could be achieved. HTPD solutions can reduce both the required amount of sample and the time needed for development of various chromatography steps.

GE Healthcare's PreDictor 96-well filter plates or PreDictor RoboColumn™ units, prefilled with BioProcess chromatography resins, are suitable for efficient high-throughput screening of both different chromatography resins and different chromatographic conditions during process development. Defined conditions can be verified and further optimized using small-scale columns. PreDictor filter plates are also available with ReadyToProcess Adsorber membranes.

More information on HTPD can be found in the handbook 28940358 (22).

Case studies

Three-step Fab purification process

A kappa subclass Fab fragment expressed in *E. coli* was used in the development of a Fab purification process. Capto L resin was selected for the initial Fab capture from *E. coli* supernatant to reduce host cell proteins (HCP) and endotoxin levels. To reduce Fab aggregates, Capto SP ImpRes, run in bind-elute mode, was selected for the intermediate purification step. Capto SP ImpRes is a high-resolution CIEX resin that allows efficient separation of aggregates from monomers. Capto Q AIEX was used to remove remaining impurities in a final polishing step. Capto Q is an excellent choice for polishing of proteins with a high isoelectric point. As the isoelectric point of the used Fab was 8.5, working at a pH of 8 or less made the Fab pass in the flowthrough, while impurities remained bound to the resin.

Process conditions were determined by using a design of experiment (DoE) approach and HTPD tools. Factors such as sample load and residence time as well as pH and conductivity in wash and elution buffers were investigated. Fab purity and yield were used as output parameters. Unit operation recoveries were all > 90%. The total process Fab recovery was 87% at an aggregate content of 0.8%. HCP and endotoxins levels were significantly reduced over the process. Protein L ligand leakage was below detectable levels. Results are summarized in Table 3.

More details about the development of this three-step Fab purification process can be found in Application note 29032066 (23).

Table 3. Summary of results for the three-step Fab purification process

Sample	Recovery (%)	Aggregates (%)	HCP (ppm)	Endotoxin (EU/mg Fab)	Protein L (ng/mL)
Feed	100	NA	440 000	1 720 000	
Capto L	97.3	3.30	13	11	<5.7
Capto SP ImpRes	93.1	0.76	8	0.05	<5.7
Capto Q	95.8	0.80	6	0.06	<5.7
Total yield	86.8				

Three-step dAb purification process

A dAb expressed in the periplasm of *E. coli* and released by heat treatment of the bacterial suspension was used in the development of a dAb purification process. Clarification of the bacterial suspension was performed in a microfiltration step using hollow fiber filters. Capto L resin was selected for initial capture for reduction of HCP and endotoxin levels. Capto MMC ImpRes, run in bind-elute mode, was selected for the intermediate purification step for its ability to efficiently reduce HCP further. Capto MMC ImpRes is a weak CIEX multimodal resin with high selectivity in a broad pH/salt window, allowing the use of the resin under a variety of process conditions to solve challenging purification tasks. For final polishing, Capto adhere ImpRes multimodal AIEX resin was used in flow-through mode. Like Capto Q, Capto adhere ImpRes is a suitable choice when purifying proteins with a high isoelectric point. Here, working at a pH of 8.5 allowed the used dAb to pass in the flowthrough, while impurities remained bound to the resin.

Optimization of process conditions was performed by using a DoE approach. Conditions for optimal dAb purity and yield were determined using Monte Carlo simulations (Fig 8). The dAb recovery of the optimized process was 89%. Results are summarized in Table 4.

More details about the development of this three-step dAb purification process can be found in Application note 29065541 (24).

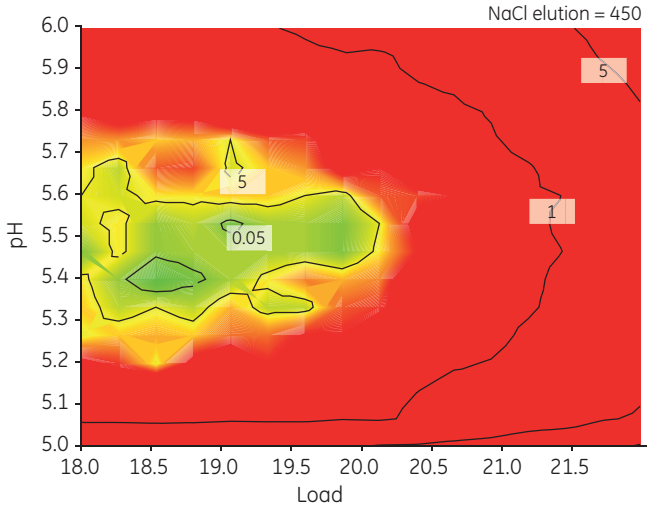


Fig 8. Monte Carlo simulation of dAb purity and yield after the initial capture step. The green surface area shows where recovery is > 83% and the HCP content is < 75 ppm. 100 000 experiments were performed *in silico*.

Table 4. Summary of results for the three-step dAb purification process

Sample	Recovery (%)	HCP (ppm)	Endotoxin (EU/mg dAb)	Protein L (ng/mL)
Feed	100	> 200 000	> 2 000 000	
Capto L	99.6	159	1.53	<LOQ
Capto MMC ImpRes	86.4	9.0	0.20	<LOQ
Capto adhere ImpRes	93.9	5.5	<0.09	<LOQ
Total yield	80.8			

Scale-up of the dAb capture step using ready-to-use products

The dAb capture step was further scaled to pilot manufacturing scale using a 2.5 L prepacked ReadyToProcess Capto L column and ready-made HyClone™ buffers. ReadyCircuit™ bags and tubing assemblies were used to enable closed system operations. Custom made cGMP manufactured HyClone buffers were delivered in single-use containers that could be directly connected to the equipment. Reproducible results from triplicate runs indicate process robustness (Fig 9). Results from the scaled-up process were comparable with those from the process run at laboratory scale (Table 5).

More details about the scale-up of the dAb capture step can be found in Application note 29227450 (25).

Column: 2.5 L ReadyToProcess Capto L columns
Equilibration: 20 mM sodium citrate + 800 mM NaCl, pH 5.0
Sample: 230 L clarified dAb containing cell culture supernatant
Sample load: 12 g/L resin
Wash 1 20 mM sodium citrate + 800 mM NaCl, pH 5.0
Wash 2: 20 mM sodium citrate, pH 5.0
Elution: 20 mM sodium citrate, pH 2.8
System: ÄKTA ready

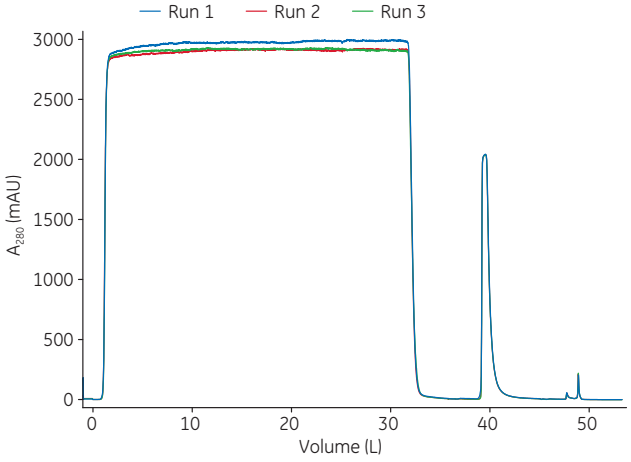


Fig 9. Overlay of chromatograms from triplicate dAb capture runs in 2.5 L ReadyToProcess Capto L columns.

Table 5. Summary of dAb yield and purity for Capto L chromatography steps

Sample	dAb recovery (%)	HCP (ppm)	Endotoxin (EU/mg dAb)
Feed	100	300 000*	66 979
HiScale™ column, eluate†	> 93	151	Not analyzed
ReadyToProcess column, eluate‡	> 90	172†	2.5

*Approximate levels. †Average of duplicate runs. ‡Average of triplicate runs.

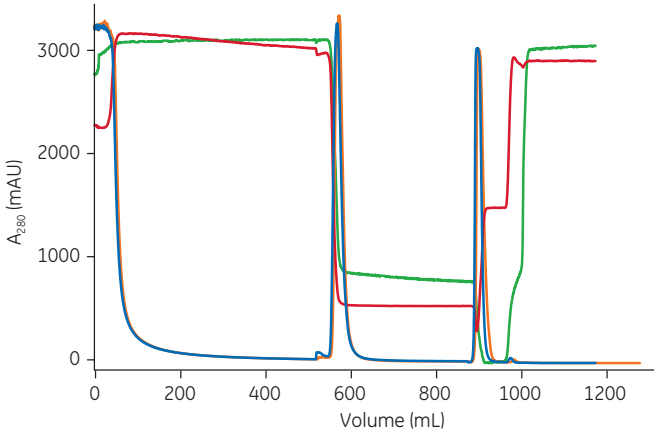
Capture of scFv fusion protein from a challenging feedstock

A human scFv fusion protein was used for development of this capture step. For capture of scFv from animal plasma, Capto L resin was selected for its high selectivity and high capacity. By optimizing process conditions, with regard to wash and elusion pH, a scFv recovery of 93% at high purity could be achieved in the developed capture step (Fig 10).

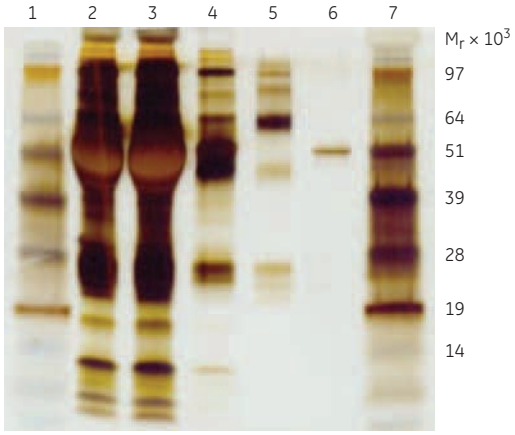
More details about the development of this scFv purification step can be found in Application note 29014456 (26).

(A)

Column: XK 26/20 packed with 34 mL Capto L, bed height 6.4 cm
Sample: Plasma containing scFv fusion protein
Loading: 6.7 mg scFv fusion protein/mL medium
Buffer A: PBS + 0.1 M glycine, pH 8
Buffer W2: 0.05 M glycine + 0.05 M citrate, pH 3.5
Buffer B: 0.05 M glycine + 0.05 M citrate, pH 2
Flow rate: 7.4 mL/min (residence time 4.6 min)
System: ÄKTA system



(B)



Lane
1. Marker
2. Sample
3. Flowthrough
4. Wash PBS
5. Wash pH 3.5
6. Elution pH 2.0
7. Marker

Fig 10. (A) Two consecutive purifications performed on Capto L using optimized wash and elution conditions. (B) SDS-PAGE results confirm the high purity of the target protein (Lane 6).

Conclusions

This whitepaper describes GE Healthcare's range of chromatography resins suitable for downstream purification of antibody fragments at industrial scale. The use of these resins in platform-based purification of multiple types of antibody fragments is discussed. Capto L resin is suitable for high-selective capture of antibody fragments containing the kappa light chain to high purity and yield in one step. KappaSelect resin binds to a different region on the kappa light chain and can be used under conditions less suitable for Capto L resin. For Fabs containing lambda light chain, LambdaFabSelect resin can be used. MabSelect resin offers a useful alternative for capture of heavy chain dAbs containing the V_{H3} domain subtype. Case studies demonstrate the high selectivity of these resins. High recovery at high purity could be achieved in the described purification processes. For efficient process development, GE Healthcare's selection of chromatography resins is available in a range of scalable formats for HTPD applications.

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